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Tumor Vascularity Is Not a Prognostic Factor for Malignant Melanoma of the Skin

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Tumor vascularity has been proposed as a prognostic indicator for a number of solid tumors. Although a correlation between microvessel number and metastatic behavior has also been suggested for cutaneous melanoma, the small number of cases studied to date allows one to draw only preliminary conclusions. In this study, we have assessed tumor vascularity in cutaneous melanoma by comparing 60 cases of metastasizing and non-metastasizing tumors matched for tumor thickness, age, sex, and anatomic site. Ulex europaeus agglutinin I appeared to be the most suitable vascular marker for this study. Our results indicate that there was no statistically significant difference between the two groups with regard to tumor vascularity. Even after identifying 15 cases of thin (<1.0 mm thick) melanoma, there was no significant difference in the number of microvessels between metastasizing and non-metastasizing tumors. Comparison of patterns of vascular microarchitecture also failed to discriminate between the two groups. Thus, our results indicate that tumor vascularity may not be an independent prognostic factor for cutaneous melanoma. (Am J Pathol 1995, 147:1049–1056)

Cutaneous melanoma is the most fatal cancer developing from the skin.[1] Its incidence is currently rising at a rate greater than for any other form of cancer in the United States.[1] The potential fatality of melanomas is primarily related to the propensity of the tumor to metastasize. Therefore, numerous investigators have attempted to identify features in cutaneous melanoma that would predict metastasis and therefore provide essential prognostic information.

Tumor thickness is currently the most reliable prognostic factor for cutaneous melanoma.[2] For example, tumor thickness <0.76 mm generally predicts a good prognosis with a 10-year survival probability of approximately 94%, whereas the prognosis of thick melanomas (thickness > 4 mm) is usually poor (10-year survival, 40%).[2,3] However, exceptions exist. A subset of thin melanomas metastasizes and some thick melanomas do not.[4–7] Therefore, several investigators have studied other parameters of potential prognostic value for melanoma.

One such potential parameter is tumor vascularity, since angiogenesis has been proposed as critical for metastasis.[8–10] Stimulated by the theory of the importance of angiogenesis in cancer metastasis, several studies have indicated that the number and density of microvessels in solid tumors such as breast,[11,12] lung,[13] and prostate carcinoma[14,15] correlate with their potential to invade and metastasize. Preliminary studies suggest similar results for cutaneous melanoma.[16,17]

However, because of the very small number of cases in these studies and conflicting reports by other investigators,[18] the prognostic significance of

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vascularity in melanomas remains unsettled. We have addressed this problem by studying 60 patients with metastatic melanoma matched with a similar group of 60 patients with non-metastasizing melanoma.

Materials and Methods

Selection of Cases

Sixty patients with primary cutaneous melanoma and documented metastasis were matched with a similar group of 60 patients with non-metastasizing primary cutaneous melanoma and mean disease-free follow-up of 8.9 years (range 5 to 22 years). The two groups of patients were matched for major prognostic factors including tumor thickness, anatomic site of the melanoma (major sites for matching included head and neck, trunk, nonacral extremities, and acral areas), age, and gender. There were no restrictions with reference to age or other histological parameters of the melanomas. The cases were retrieved from the following institutions: Brigham and Women's Hospital; Massachusetts General Hospital; the University of Aberdeen, UK; University of California at Los Angeles; University of Pennsylvania; Sidney Melanoma Unit, Australia; Wake Forest University; and the institutions cooperating with the Cancer Prevention Research Unit at Yale University.

Quantification of Tumor Vascularity

Tumor microvessels were evaluated in sections from formalin-fixed, paraffin-embedded material from the archives of the institutions listed above. Sections were stained with an antibody against von Willebrand factor (anti-factor VIII-related antigen (FVIII RA)), (DAKO Corp., Carpinteria, CA, dilution 1:25), antibody against CD34 (Immunotech, Inc., Westbrook, ME, dilution 1:200) or using the lectin Ulex europeus agglutinin I (UEAI) (Vector Laboratories, Burlingame, CA, dilution 1:80) by means of the avidin-biotin complex method and the amino ethyl carbazole chromogen. In each case, the melanoma was scanned microscopically for the area judged to have the greatest vascular density. In the majority of cases, many "hot spots" of tumor vascularity were seen at the periphery, ie, the advancing edge of the tumors. The number of microvessels per microscopic field (400 × magnification) was recorded, using the same microscope (Olympus BH2, Dexter Instruments, San Antonio, TX).[19,20] Structures were counted as microvessels, if they stained positively with a vascular marker and morphologically appeared vascular, ie, had a lumen surrounded by endothelium. Tumor vascularity was recorded independently by two observers (KJB and RLB) without knowledge of clinical outcome. Intra-observer reproducibility has been previously reported.[19] Inter-observer reproducibility was analyzed by the Pearson correlation coefficient for independent microvessel counts in 20 cases. The significance of differences in mean or highest vessel count between samples was evaluated by Student's *t*-test for independent samples.

In a subset of cases, tumor vascularity was also studied using a computerized image analysis system (Bioquant Systems IV, R&M Biometrics, Inc., Nashville, TN) to quantify the numbers of microvessels and percentage of area stained with a vascular marker (PVA).[19] The reproducibility of both microvessel counts and PVA by image analysis was tested as was the correlation between numbers of microvessels quantified by image analysis as opposed to manual counting.

Analysis of Vascular Patterns

Distinctive vascular patterns associated with melanomas were also studied for prognostic significance. In brief, these patterns included: 1) the "normal" pattern, in which the architectural distribution of vessels within or surrounding the tumor does not differ from areas of the skin within an excisional biopsy distant from the tumor; 2) the "cluster" pattern, in which randomly arranged groups of capillaries, with and without branching, form clusters of microvessels; 3) the "diffuse" pattern, in which there is a discernible increase in peri- and/or intratumoral vascular density compared with adjacent uninvolved skin (the increased vascularity is diffuse within and/or around the tumor and lacks a distinctive architectural distribution of vessels); and 4) the "arcade" pattern, in which a network of capillaries is distributed in a circular or near circular fashion around tumor nodules (when such circles or semicircles intersect in two-dimensional histological sections, they appear to form "arcades").

Results

Comparison of Vascular Markers

Three different vascular markers, FVIII RA, CD34, and UEAI were compared in their sensitivity to iden-

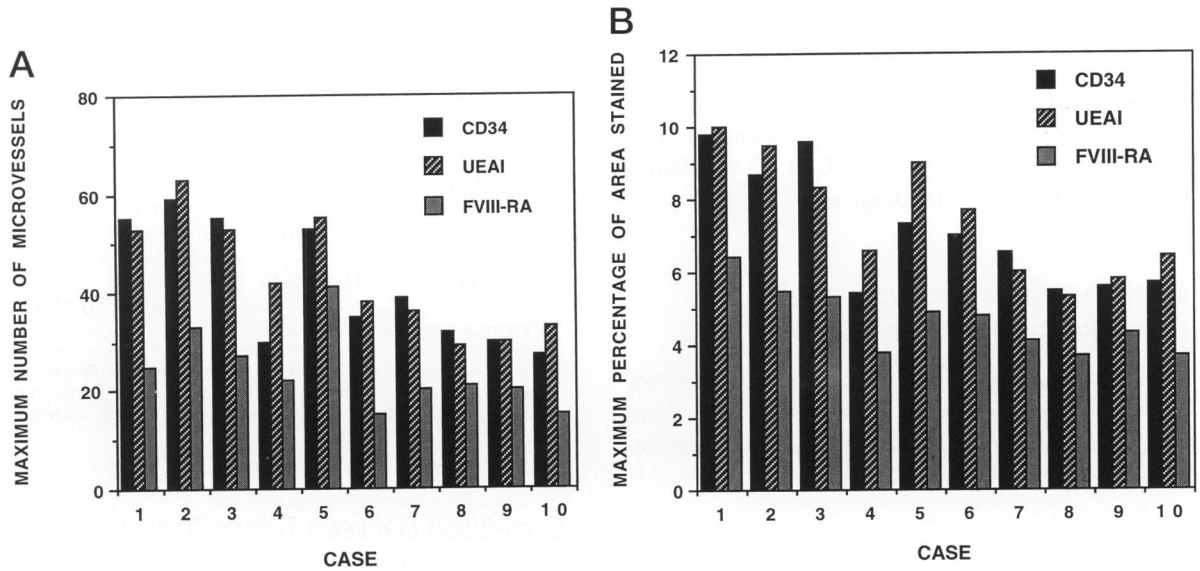

Figure 1. *10 cases of cutaneous melanoma were stained immunohistochemically, using three different vascular markers (CD34, UEAI, and FVIII RA). The one field (400×) with the highest number of microvessels out of five fields counted per case is shown in* A. B *illustrates the one field out of five examined fields with the highest percentage of area stained, using computer-assisted image analysis.*

tify microvessels in 10 cases of human cutaneous melanomas by immunohistochemistry. Five areas of highest microvessel density were chosen per tumor. Figure 1 shows the results on the one microscopic field (400×) with highest vascularity per case (Figure 1A, number of microvessels counted; Figure 1B, percentage of area stained, using computerized image analysis). Figure 2 describes the results on the average of five microscopic fields (400×, each) per case (Figure 2A, number of microvessels counted; Figure 2B, percentage of area stained, using computerized image analysis). The data show that CD34 and UEAI are equally sensitive in the detection of microvessels. Both of the latter markers were approximately 1.6 times more sensitive than FVIII RA, when image analysis was used. They were 1.9 times more sensitive than FVIII RA, when the number of microvessels was counted. Because in our hands UEAI shows less background staining for non-vascular structures in the skin than does CD34, we chose to use UEAI to examine vascularity in the remainder of this study.

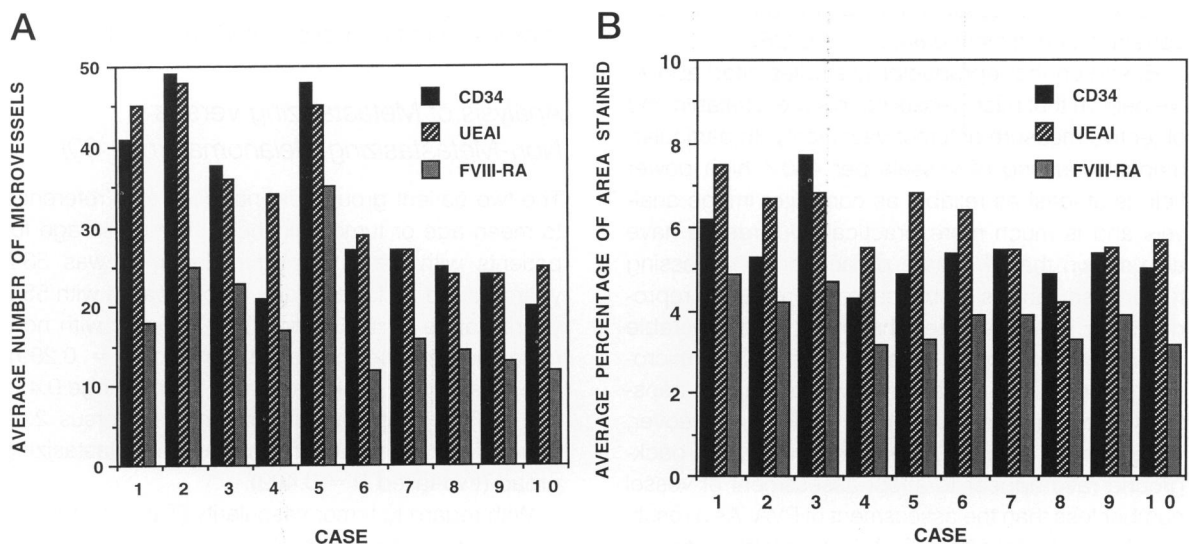

Figure 2. *10 cases of cutaneous melanoma were stained immunohistochemically, using three different vascular markers (CD34, UEAI, and FVIII RA). Five fields judged to contain the highest number of microvessels were examined per case. The average number of microvessels per 400× microscopic fields is shown in* A. B *illustrates the average percentage of area stained, using computer-assisted image analysis.*

Intra-Observer Reproducibility of Conventional Microvessel Counts

As previously reported,[19] we have verified intra-observer reproducibility for microvessel counts (Pearson correlation coefficient 0.94, $P = 0.0001$) from two independent readings of 10 cases.

Inter-Observer Reproducibility of Conventional Microvessel Counts

Independent readings of 20 cases by two observers (KJB, RLB) revealed significant reproducibility of microvessels by manual counting (Pearson correlation coefficient 0.84, $P = 0.000004$).

Reproducibility of Microvessel Counts and PVA by Image Analysis

Two independent, blinded analyses of 10 cases for microvessel counts and PVA revealed that microvessel counts were reproducible (Pearson correlation coefficient 0.57, $P = 0.02$) but PVA was not reproducible (Pearson correlation coefficient 0.27, $P = 0.32$) in our hands.

Reproducibility of Microvessel Counts Between Computer Image Analysis and Conventional Counting

As previously reported,[19] there was a statistically significant correlation between these two methods of recording microvessel counts for 10 cases (Pearson correlation coefficient 0.84, $P = 0.0025$).

Based on the reproducibility studies cited above, we believe that microvessel counts are a reliable and objective measure of tumor vascularity. In particular, manual counting of vessels per 400× high power field is at least as reliable as computer image analysis and is much more practical. Our results have also shown that PVA as a parameter for assessing tumor vascularity is problematic with regard to reproducibility. We have noted that there is considerable variation in caliber of vessels in any given microscopic field. This variance in vessel caliber translates into significant variability of PVA. Moreover, variations in staining intensity or signal-to-background ratio appear to affect assessment of vessel number less than the assessment of PVA. As a result, we do not believe that PVA is a reliable index of tumor vascularity (at least in our studies) and have abandoned the use of this parameter.

Table 1. *Mean and Highest Microvessel Numbers, Metastatic versus Disease-Free Melanomas*

Condition of melanomas	Microvessel number	SD	*P* values	
			One-tailed	Two-tailed
Metastatic	30.2 (mean)	13.5	0.1163	0.2326
Disease-free	33.2 (mean)	13.5		
Metastatic	35.9 (highest)	15.8	0.2228	0.4456
Disease-free	38.1 (highest)	16.2		

Comparison of mean (of five 400× microscopic fields) and highest microvessel numbers between metastatic and non-metastatic melanomas (n = 60 each), matched for thickness, age, and sex.

Correlation Between Tumor Thickness and Microvessel Density

Because tumor thickness is considered the most important prognostic factor for cutaneous melanoma, microvessel density was compared with tumor thickness in all metastasizing and non-metastasizing melanomas (n = 120). The Pearson correlation coefficients were 0.0502 ($P > 0.95$, two-tailed) for tumor thickness *versus* mean microvessel density, and 0.0740 ($P > 0.95$, two-tailed) for tumor thickness *versus* single highest microvessel number, respectively. The Pearson correlation coefficient measures the degree of concordance between two matched (paired) samples. A coefficient may theoretically vary from 0 (indicating no relationship between the samples) to 1 (indicating a highly correlated relationship). The coefficients calculated here indicate no significant relationship between tumor thickness and tumor-associated vascularity.

Analysis of Metastasizing versus *Non-Metastasizing Melanomas (n = 60)*

The two patient groups did not differ with reference to mean age or tumor thickness. The mean age for patients with metastasizing melanomas was 53.2 years (range 27 to 78 years) as compared with 55.9 years (range 21 to 84 years) for patients with non-metastasizing melanomas (two-tailed $P = 0.299$). The mean tumor thickness was 2.2 mm (range 0.4 to 7.4 mm) for metastasizing melanomas versus 2.00 mm (range 0.3 to 5.9 mm) for the non-metastasizing group (two-tailed $P = 0.499$).

With regard to tumor vascularity (Table 1), the two groups showed no significant differences for either mean numbers of microvessels or highest microvessel counts. Patients with metastatic melanomas had

Table 2. *Mean and Highest Microvessel Numbers, Metastatic versus Disease-Free Thin Melanomas*

Condition of melanomas	Microvessel number	SD	*P* values One-tailed	*P* values Two-tailed
Metastatic	33.47 (mean)	14.1	0.1163	0.2326
Disease-free	33.13 (mean)	11.7		
Metastatic	38.7 (highest)	15.6	0.3116	0.6232
Disease-free	36.1 (highest)	13.8		

Comparison of mean (of five 400× microscopic fields) and highest microvessel numbers between metastatic and non-metastatic thin (<1.0 mm thick) melanomas (n = 15 each), matched for thickness, age, and sex.

mean microvessel and highest microvessel counts of 30.2 and 35.8, respectively, *versus* 33.2 and 38.1, respectively, for patients who were disease-free (two-tailed $P = 0.233$ and 0.446 for mean and highest microvessel counts, respectively). We also found no significant differences between the two groups using a computerized image analysis system (Bioquant Systems IV, R&M Biometrics, Inc.) measuring the PVA per 400× microscopic filed (data not shown).

Microvessel Number in Thin Melanomas (n = 15)

A subgroup of thin melanomas (<1.0 mm) was analyzed to evaluate whether vascularity could discriminate between metastasizing and non-metastasizing tumors (Table 2). The two groups of 15 patients each did not differ significantly with reference to mean age or tumor thickness. The mean age for patients with metastasizing melanomas was 52.2 years as compared with 54.2 years for non-metastasizing melanomas (two-tailed $P = 6.65$). The mean tumor thickness was 0.760 mm for metastasizing melanomas and 0.756 mm for non-metastasizing groups with regard to tumor vascularity for either mean numbers of microvessels or highest microvessel count. The mean and highest microvessel counts in patients with metastatic tumors were 33.47 and 38.73, respectively, *versus* 32.13 and 36.07, respectively, in disease-free patients (two-tailed $P = 0.623$ for both mean and highest microvessel number).

Analysis of Vascular Patterns

The most commonly encountered pattern was diffuse hypervascularity (48%), followed by the arcade (19%), normal (17%), and cluster pattern (16%) (See Table 3). None of the four vascular patterns was predictive of metastases.

Table 3. *Frequency of Microvascular Architectural Patterns in Metastatic versus Non-Metastatic (Disease-Free) Melanomas*

Vascular patterns	Metastatic	Disease-free
Normal	12	7
Clusters	9	11
Diffuse	27	31
Arcades	12	11

n = 120; 60 matched cases.

Discussion

The role of tumor vascularity as a prognostic indicator in cutaneous melanoma is an unsettled issue. Preliminary studies by a number of investigators have suggested that there might be a relationship between the extent of tumor vascularity and incidence of metastases in cutaneous melanoma.[16,17] However, the data available thus far are controversial. Srivastava et al[16] reported that increased vascularity at the base of the tumor, as measured by percent vessel area, may have prognostic significance in intermediate thickness (0.76 to 4.0 mm) melanomas. Recently, Graham et al[17] found that microvessel number correlated with metastatic behavior in thin (<0.76 mm) melanomas, while it failed to predict clinical outcome in thick (> 4.0 mm) tumors. However, the results of both these studies must be interpreted with caution because of the small number of cases investigated.

In this study, we investigated the prognostic role of tumor vascularity in cutaneous melanoma. Because tumor-dependent neovascularization is believed to manifest as "hot spots" of high capillary density,[11,19] we first searched for the most suitable vascular marker to identify such regions of high vascular density in human skin. Comparing three different vascular markers in their sensitivity to visualize microvessels, both UEAI and CD34 were found to be superior to FVIIIRA. These results are in agreement with previous reports, in which CD34 and UEAI were each separately compared with FVIIIRA, but not directly with each other.[22–25] Our observations and those of others suggest that the improved sensitivity of UEAI and CD34 appears primarily related to their ability to stain very small capillaries and venules better than FVIIIRA. It is difficult to exclude that some lymphatic vessels stain positively with UEAI and/or CD34. However, the vast majority of peri- or intratu-

moral microvessels on which the comparison of vascular markers was performed were morphologically endothelial-lined capillaries. While CD34 and UEAI were approximately equally sensitive in demonstrating microvessels, in our hands there was less nonspecific staining with UEAI than with CD34. Most recent studies concerning tumor vascularity in melanomas have used either UEAI or CD34.[16,17] Because the latter markers are equally sensitive, the choice between them should have little influence on differences in results between these studies.

Using UEAI as vascular marker, we then measured regions of high microvessel density in 60 patients with metastasizing melanoma matched with a comparable group of nonmetastasizing melanoma. To our knowledge, this is the largest study on this subject to date. Our findings strongly suggest that tumor vascularity is not an independent prognostic factor for melanoma, regardless of tumor thickness. This result contrasts with the above-mentioned studies.[16,17] Although there were some differences in the methodology of quantifying vascularity, the major difference appears to lie in the number of cases studied. Srivastava et al[16] examined 10 cases of metastatic/recurrent cutaneous melanoma of intermediate thickness matched with 10 cases with no subsequent recurrence or metastasis. Graham et al[17] based their conclusions on the comparison of five matched cases of thin melanomas, a limitation in number of which the authors were well aware.

The lack of prognostic significance of tumor vascularity suggested by our results applies primarily to tumors measuring >1.0 mm in thickness, because the majority of cases studied (77%) were thicker than 1.0 mm. This finding is no surprise. In a previous study, and again in this current study, we failed to detect any appreciable change in tumor vascularity with greater tumor thickness, although thickness is currently still the most reliable prognostic indicator.[19] While 15 cases may not be sufficient for a verdict on tumor vascularity in thin melanomas, our findings provide substantial evidence against its potential prognostic value.

Rather than microvessel numbers, some investigators have studied vascular patterns surrounding tumors. It has been suggested that neovascularization induced by tumors manifests an altered vascular microarchitecture surrounding the tumor, which may contain prognostic information. In uveal melanomas, distinct vascularization patterns have correlated with clinical outcome.[26–28] Therefore, we have also attempted to identify vascular patterns that might discriminate metastasizing from non-metastasizing tumors. In our study, none of the patterns examined had prognostic significance. However, those vascular patterns (at least one closed vascular loop or a network of loops) that in uveal melanomas were strongly associated with metastases were not clearly developed in the cutaneous melanomas in our series. Thus, it remains a possibility that in a subset of cutaneous melanoma, closed vascular loops or another yet unidentified pattern of peri-/intratumoral vascularity might yield prognostic information.

Although there is ample evidence that increased vascular supply and vascular access by intra- or peritumoral neovascularization may facilitate tumor growth and metastasis,[10,21] the diagnostic and prognostic value of angiogenesis remains controversial. At least as far as cutaneous melanomas are concerned, our results indicate that it may be too simplistic to assume that more microvessels mean higher metastatic probability.

Our inability to correlate microvessel number with metastatic potential may have intrinsic biological and/or methodological reasons. The likelihood of a tumor metastasizing is dependent upon a large number of poorly understood factors, which among others include its capacity for vascular invasion, evasion of the immune system, and homing to an environment suitable for survival.[9] If there is great diversity among individual melanomas with regard to their capacity to pursue each particular step required for metastasis, it may be impossible to evaluate the precise role of tumor vascularity. Although melanomas can be matched for known prognostic factors such as thickness, they cannot be standardized by currently available means with reference to cellular heterogeneity and degree of differentiation, intrinsic potential to pursue various steps of metastasis, or immunocompetence of the host. Furthermore, many factors not related to the biology of an individual tumor, such as exogenous trauma or venous stasis, may influence peritumoral vascularity.

A major methodological problem relates to our inability to discriminate between vascular structures, in particular our inability to selectively stain lymphatic vessels. Yet, lymphatic spread probably represents the major route of metastasis for many tumors, whose metastatic potential was said to be reflected by an increase in vascularity. It is also possible that only a subset of functionally distinct new microvessels are used for metastasis, but we cannot selectively identify them by currently available means.

A last major consideration relates to histological regression, which represents an intrinsic problem for evaluating the prognostic potential of vascularity in thin melanomas. Regressive changes have been associated with a poorer prognosis in thin melano-

mas.[5–7] It would seem necessary to match cases for the extent of regression, especially since Barnhill and Levy[20] reported greater vascularity in thin melanomas with regression than in those lacking such features. Although there is currently no consensus concerning criteria for the quantification and therefore standardization of regression, recent evidence suggests regression may be reliably recognized when objective guidelines are formulated.[29] This makes it difficult to compare thin melanomas with regard to vascularity, once regressive changes are noted.

Although our preliminary results for thin melanomas do not indicate that microvessel number has prognostic significance, we do not believe that the number of cases studied so far is sufficient to settle the issue. This subset of melanomas requires further studies, since such melanomas are being diagnosed with increasing frequency.

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